

**REMARKS**

This Amendment is respectfully submitted in response to the Office Action rendered June 28, 2001. It is timely submitted in view of the Petition for Extension of Time submitted concurrently herewith.

Claims 26-28 have been added in order to describe certain embodiments of the compositions of applicants' invention. They find basis in the Specification at page 10, lines 6-8, page 10, lines 14-16, page 10, lines 18-28, page 11, lines 1-2 and page 11, line 16. A marked-up copy of the claims is submitted concurrently herewith as an Appendix to this Amendment.

Reference to the provisional patent applications from which the above-captioned patent application claims the benefit of priority under 35 U.S.C. 119(e) has been added to the Specification. No new matter has been added as these applications are referred to in the cover letter for the non-provisional application.

The Office Action of June 28, 2001 rejected claims 1-12 and 15-25 under 35 U.S.C. 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Office Action noted that the Markush group set forth in claims 1 and 25 had the term "or" rather than the term --and--. In order to correct this inadvertent error and restore proper Markush terminology to the claims, applicants have amended claims 1 and 25 to include the term --and--. Applicants respectfully request reconsideration of this rejection in view of this correction.

The Office Action of June 28, 2001 rejected claims 1-10 and 12-25 under 35 U.S.C. 103(a) as being unpatentable over either De Stoutz (U.S. 5,503,832) or JP 62036394 (Abstract) in view of Bissett et al. (U.S. 6,183,761) and Knight et al. (U.S. 6,017,549). Applicants respectfully request reconsideration of this rejection in light of the ensuing discussion, the publications submitted herewith and the Declaration of Jonathan Miller submitted herewith.

The basis for the rejection was as follows:

De Stoutz teaches using soymilk in skin care products and other cosmetic products...JP 62036304 ...teaches using soybean milk in cosmetic compositions for skin and hair. Neither reference explicitly teaches a stabilizing system of the instant invention. However, the claimed stabilizing system comprising an antioxidant...a chelating agent...and/or a preservative...is known and widely used in the art of

cosmetic compositions for the purpose of stabilizing active ingredients...[Office Action, 6/27/01, p. 3]

Applicants respectfully submit that neither De Stoutz nor JP 62036394 suggests or describes the compositions or methods of applicants' invention, as pointed out in the Office Action. De Stoutz relates to "a method for extracting the soluble material from oil-bearing beans or seeds and, more particularly with a method for manufacturing a 'milk' or creams based on such oil-bearing seeds." [De Stoutz, Col. 1, l. 6-10]. However, De Stoutz actually teaches away from the compositions of applicants' invention, as it suggests that "nutritionally undesirable" factors should be reduced, i.e., denatured, during :

Also, because of the very short contact time of the seeds with water which is preferably at 80° C., a considerable decrease in the bacterial load is achieved, as well as **in the amount of factors which, in the case of food products, are nutritionally undesirable (antitrypsin factors)**. [Col. 2, l. 59-63] (emphasis added)

These "nutritionally undesirable" factors, antitrypsin factors (also known as trypsin inhibitory factors), are the very measure of biological activity which applicants' state as marking a "nondenatured" composition according to their invention [See Specification, page 7, lines 12-14]. In contrast, De Stoutz thus suggests that pre-soaking tends to increase the amount of enzymes in the final product. In fact, Fig. 3 of De Stoutz demonstrates the **reduction** of antitrypsin content without pre-soaking, indicating that including such compounds in the final product is undesirable. Even in cosmetic products, De Stoutz suggests that the compositions be subjected to a heat treatment, which tends to denature proteins such as trypsin inhibitors:

Clearly, for a proper conservation of the products obtained, when the soy milks and creams are designed for use in skin care, it may be necessary to subject these products to a thermal treatment. This thermal treatment can advantageously be carried out using the Joule effect according to the method described in EP 0.476.311. [Col. 4, l. 12-17]

Thus, De Stoutz neither suggests nor describes the compositions or methods of applicants' invention.

Nor does the JP 62036304 publication suggest or describe the compositions of applicants' invention. The JP 62036304 publication merely indicates that cosmetics containing soya milk may be used for cleaning and moisturizing the skin and hair. Nowhere does it suggest or describe the stable compositions or novel methods of applicants' invention. At the time of the JP 62036304 publication, soy products containing was known to cause pancreatic

enlargement and intestinal discomfort. As set forth in the articles appended hereto, at the time of the JP 62036304 publication, it was desirable to treat soy products so as to denature the proteins contained therein [See "Inhibition of Human and Rat Pancreatic Proteinase by Crude and Purified Soybean Proteinase Inhibitors, Krogdahl et al., *J. Nutr.* 109: 551-558, 1979; "Structure-Function Relationships of proteinase Inhibitors from Soybean (Bowman-Birk) and Lima Bean", E. Kay, *The Journal of Biological Chemistry*, Vol. 254, No. 16, Issue of August 25, pp. 7648-7650, 1979; and "The Effects of Soybean Trypsin Inhibitors on the Pancreas of Animals and Man: A Review", D. F. Flavin, *Vet Hum Toxicol.* 24, pp. 25-28, 1982]. Nowhere does JP 62036304 suggest or describe a **different** treatment for the soy-containing products it mentions in order to retain the presence of soy trypsin inhibitor or other proteins that were known to be dangerous when ingested.

In contrast, the methods and compositions of applicants' invention contain **non-denatured** soy products. As set forth in the Specification at page 12, line 15 through page 14, line 27, the applicants' have found that soy proteins such as Soy Trypsin Inhibitor act, for example, to depigment skin, reduce skin oiliness and shine, treat or prevent the condition of acne, relieve pain and burning after sun exposure even the tone and texture of skin, and increase elastic properties of skin. Neither De Stoutz patent nor the JP 620363304 abstract describes or suggests either non-denatured soy- or legume-containing compositions nor these methods of using such compositions to treat or prevent the aforementioned skin conditions.

Nor do the Bissett et al. or Knight et al. patents remedy the deficiencies of De Stoutz and/or JP 62036304 in rendering the compositions of applicants' invention obvious. Bissett et al., for examples, relates to compositions containing a vitamin B3 compound and certain specific compounds such as flavanones. It lists at least two dozen "optional components" that may be included in these compositions, but nowhere suggests or describes the soy product-containing compounds of applicants' invention. Bissett et al. neither recognizes nor solves the problem of formulating soy- or legume-containing compositions as to maintaining non-denatured products in a physically or chemically stable formulation. Rather, Bissett et al. focuses on vitamin B3 compounds and compounds that may be extracted from soy products, but may also be synthesized [Col. 9, l. 13-17]. Furthermore, the "optional components" set forth at col. 10, l. 47-50 of Bissett et al. are described as **optional**, i.e. they need not be present in the composition. Thus, one of ordinary skill in the art would not

have looked to Bissett et al. to solve the stability problems of formulating with non-denatured soy protein products, particularly of soy milk.

Knight et al. was cited as containing caffeine powder. However, while the compositions of applicants' invention may contain caffeine as an anti-cellulite active ingredient, nothing in Knight et al. would have directed one of ordinary skill in the art toward formulating caffeine compositions with non-denatured soy protein products in a manner similar to the compositions of applicants' invention. Knight et al. neither recognizes such a possibility nor suggests a means for solving problems ensuing therefrom.

That it was not clear to one of ordinary skill in the art how to solve the physical and chemical stability problems of formulating with non-denatured soy protein products is set forth in the accompanying Declaration of Jonathan D. Miller. As demonstrated therein, during the course of developing compositions containing non-denatured soy products that retained their biological activity and were physically and chemically stable, Compositions A and B were formulated [Declaration of Jonathan D. Miller, ¶¶2, 3]. These compositions both failed to meet physical and chemical stability requirements for cosmetic compositions.

Composition A, for example, contained certain of the "optional components" set forth in Bissett et al., such as ascorbyl glucoside, an ascorbic acid derivatives, octyl methoxycinnamate, a sunscreen, retinol, and vitamin E (tocopherol). Yet, is resulted in a "chunky, lumpy, physically unstable product" (Declaration of Jonathan D. Miller, ¶5). Composition B also contained certain compounds set forth in Bissett et al. at col. 10, l.19-36 and l. 40-57, such as salicylic acid, and ethanol. Composition B, too, was physically unstable as it "resulted in a sticky, clumpy product indicating that the product would not be usable as a topical cosmetic product" (Declaration of Jonathan D. Miller, ¶5. Rather, formulating the compositions of applicants' invention presented challenges due to the fact that not all thickeners and emulsifiers are compatible with non-denatured soy products (Declaration of Jonathan D. Miller, ¶6).

Neither Bissett et al. nor Knight et al. recognized these problems encountered in formulating non-denatured soy products, nor did either suggest or describe solutions for such problems. Indeed, when attempting to formulate such products utilizing some of the described elements of cosmetic compositions, the resulting compositions could not even be measured for chemical stability, due to their complete lack of physical stability.

Furthermore, none of the cited references suggests or describes compositions containing no more than 0.1% of a surfactant, as set forth in the embodiment of applicants' invention described in claim 2. In fact, JP 62036304 specifically suggests the use of more than 2% of a surfactant in soy milk compositions [See JP 62036304, Abstract]. Thus, the composition of claim 2 would not have been obvious in view of JP 62036304. As set forth in the Declaration of Jonathan D. Miller, formulating compositions containing non-denatured soy products was extremely difficult and even compositions containing conventional ingredients in conventional ranges were not successful.

Applicants therefore request reconsideration of the rejection under 35 U.S. 103(a) in view of De Stoutz or JP 62036304 in light of Bissett et al. and Knight et al. Not only does De Stoutz and JP 62036304 teach away from the compositions and methods of applicants' invention, the combination of these references with Bissett et al. and Knight et al. do not lead one of ordinary skill in the art toward the compositions and methods of applicants' invention. Not only would one of ordinary skill in the art not utilize non-denatured soy products in skin care products, based upon De Stoutz and JP 62036304, but they would not be able to formulate them into chemically or physically stable compositions using the descriptions set forth in Bissett et al. and Knight et al.

Claims 1-9, 11 and 13-25 were rejected under 35 U.S.C. 103(a) as being unpatentable over Lee et al. (KR 9208853) in view of Bissett et al. and Knight et al. The basis for this rejection was that Lee et al. "teach[es] using soybean powder in cosmetics" [Office Action of 6/28/01, p. 4]. Applicants respectfully request reconsideration of this rejection in light of the discussion herein.

Lee et al. relates to a method of preparing soybean powder that includes selecting the soybeans, heating the soybeans, cutting the soybean grain, peeling the grain, steaming the peeled grain, drying and pulverizing the grain [Lee et al., Abstract]. While Lee et al. mentions that "the obtd. soybean powder is useful for the prodn. of food, cosmetic, etc." [Lee et al., Abstract], nowhere does Lee et al. suggest or describe the compositions or methods of applicants' invention.

Lee et al. actually suggests at least two steps in treating the soybeans that would denature the protein content of the beans: heating and steaming. Nowhere does Lee et al. suggest or describe steps that would preserve the trypsin inhibitory activity of the composition. In fact, in setting forth that the resulting powder would be useful in the

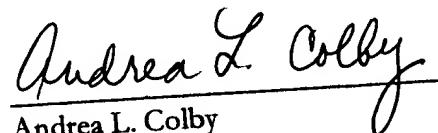
production of food and cosmetics, and in light of the contemporaneous knowledge that non-denatured soybean products were harmful, Lee et al.'s process would necessarily have included the denaturation of the enzymes in the soybean. Thus, Lee et al. teaches away from the compositions and methods of applicants' invention.

Nor do Bissett et al. or Knight et al. remedy the deficiencies of Lee et al. in leading one of ordinary skill in the art toward the compositions and methods of applicants' invention. As set forth above and in the accompanying Declaration of Jonathan D. Miller, following the descriptions set forth in Bissett et al. or Knight et al. would not result in physically or chemically stable compositions, even were they to suggest the possibility of formulating non-denatured soy powder or milk. Applicants therefore respectfully request reconsideration of the rejection of claims 1-9, 11 and 13-25 under 35 U.S.C. 103(a) as being unpatentable over Lee et al. (KR 9208853) in view of Bissett et al. and Knight et al.

An Information Disclosure Statement and additional patents and publications are being submitted under separate cover for the consideration of the Patent and Trademark Office.

On the basis of the foregoing amendments to the claims and discussion, applicants respectfully request reconsideration of the rejections set forth in the Office Action of June 28, 2001 in light of the foregoing discussion, articles and accompanying Declaration of Jonathan D. Miller. Consideration of new claims 26-29 is respectfully requested. An early allowance is earnestly solicited.

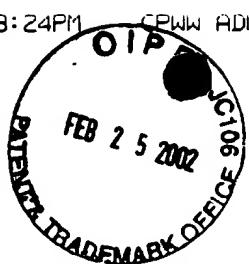
Respectfully submitted,

  
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## APPENDIX

1. (Amended) A skin care composition for the topical delivery of a soy product comprising a non-denatured soy product and a stabilizing system, said stabilizing system comprising a member selected from the group consisting of an antioxidant, a chelating agent [or] and a preservative.
25. (Amended) A skin care composition for the topical delivery of a legume product comprising a non-denatured legume product and a stabilizing system, said stabilizing system comprising a member selected from the group consisting of an antioxidant, a chelating agent [or] and a preservative.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Miri Seiberg, et al.

Serial No.: 09/698,454 Art Unit: 1616

Filed: October 27, 2000 Examiner: M. Lamm

For: **SOY DEPIGMENTING AND SKIN CARE COMPOSITIONS**

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DECLARATION OF JONATHAN D. MILLER

I, Jonathan D. Miller, hereby declare:

1. I am a named inventor of U.S. Patent Application Serial No. 09/698,454, filed October 27, 2000. I am employed by Johnson & Johnson Consumer Companies, Inc. in the position of Senior Scientist and Go-to-Market Leader. I received a Bachelor of Science degree in Chemical Engineering from Cornell College of Engineering in 1999. After receiving my Bachelor of Science degree I became employed by Johnson & Johnson in 1999. Since then, my duties have centered on the development of new skin care products in the Department of Research and Development

2. I participated in developing compositions containing non-denatured soy products that retained their biological activity and were physically and chemically stable. During the course of developing such soy-containing products, at my direction, studies were conducted to determine the physical and chemical stability of chemical compositions containing non-denatured soy.

3. At my direction, Compositions A and B were formulated. Compositions A and B contained the following ingredients:

Composition A:

<u>Excipient</u>	<u>Concentration, %w/w</u>
Deionized Water	70.58
Glycerin	3.00
Preservative	0.73
Ascorbyl glucoside	2.00
Panthenol	0.50
Carbomer	0.40
Acrylates/C <sub>10-30</sub> Alkyl Acrylates	0.25
Cross-Polymer	

Preservative	0.52
Disodium EDTA	0.10
Ascorbic Acid	0.01
C <sub>12-15</sub> Alkyl Benzoate	4.00
Octyl Methoxycinnamate	4.00
Cetyl Alcohol	1.00
Octyl hydroxystearate	1.00
Dimethicone	1.00
Steareth-10	0.50
Tocopheryl acetate	0.50
BHT	0.10
Soymilk Powder	3.50
Sodium hydroxide 10%	5.05
Green Tea Extract	1.00
Vitamin E	0.05
Retinol	0.21

Composition B:

<u>Excipient</u>	<u>Concentration, %w/w</u>
Deionized Water	56.80
Ethanol	20.00
Glycerin	3.00
Butylene Glycol	5.00
Propylene Glycol	3.00
Preservative	1.00
Salicylic acid	2.00
Soymilk Powder	5.00
Disodium EDTA	0.20
Hydroxypropylcellulose	1.50
Sodium Hydroxide (20%)	2.50

4. Composition A was an oil-in-water emulsion in the form of a lotion. Composition B was a gel formulation.

5. I observed the results of formulating Compositions A and B. Neither attempt was successful in obtaining a physically stable cosmetic formulation. Composition A resulted in a chunky, lumpy, physically unstable product. The phases of the composition separated and were not usable as a cosmetic. Composition B resulted in a sticky, clumpy product indicating that the product would not be usable as a topical cosmetic product.

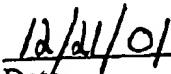
6. The formulations of Compositions A and B contained excipients known to those of ordinary skill in the art of formulating cosmetic products at the time the invention was made. Although one of ordinary skill in the art would have expected that such compositions would have been physically stable and usable as topical cosmetics,

surprisingly, they did not result in stable, usable compositions. Rather, formulating compositions using non-denatured soy products presented exceptional challenges, including: identifying thickening agents and emulsifying agents compatible with non-denatured soy that still maintained the aesthetics required for a topical cosmetic product and developing the processes necessary to incorporate the non-denatured soy into the topical composition without damaging the soy but still maintaining physical stability.

7. I conclude from the foregoing attempts to make physically stable compositions containing non-denatured soy products that one of ordinary skill in the art at the time the invention was made, using known cosmetic excipients, would not have necessarily been able to make physically stable, usable topical cosmetic compositions. It would, therefore, have been surprising and unexpected to obtain physically stable compositions containing non-denatured soy products.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

  
\_\_\_\_\_  
JONATHAN D. MILLER

  
\_\_\_\_\_  
Date

09/698,454

## Inhibition of Human and Rat Pancreatic Proteinases by Crude and Purified Soybean Proteinase Inhibitors

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**ABSTRACT.** Effects of proteinase inhibitors on total proteolytic activity and trypsin and chymotrypsin activity in human pancreatic juice were determined separately. Purified inhibitors as well as crude extracts of raw soybeans completely inhibited trypsin and chymotrypsin activity while 40 to 50% of the total proteolytic activity remained. Inhibition experiments with 1,10-*o*-phenanthroline showed that this residual proteolytic activity was due mainly to carboxypeptidase A and B. Comparative studies with rat pancreatic enzymes demonstrated certain similarities between the corresponding enzymes from rat and man. However, differences were revealed which indicate that the rat enzymes must be used with great caution when applied as models for the human proteinases when studying effects of soybean inhibitors. *J. Nutr.* 109: 551-558, 1979.

**INDEXING KEY WORDS** pancreatic proteinases · soybean inhibitors · human nutrition

In the early 1960's a program of experimental cultivation of soybeans was launched in Tanzania. The object was to produce a cheap and stable source of protein and energy which could be grown and utilized in rural areas and which was also suitable for infants and young children. Holm et al. (1) examined heat treated soybean meal from three different varieties of soya (*Glycine max*) grown in Tanzania. The results obtained in nitrogen balance experiments in rats were very different from those predicted from the amino acid patterns and this discrepancy was associated with the presence of proteinase inhibitors.

A number of inhibitors which possess different heat resistances and enzyme specificities are known to occur in soybeans. The inhibitory character of one particular variety of soybean is dependent partly on hereditary and partly on environmental factors (2, 3). The danger of variable and inadequate heat treatment is very real when the meal is produced at the village level (1). Substantial inhibitory activity

may therefore be suspected. However, the *in vivo* effect of proteinase inhibitors in soybean meal used as human food is not well understood.

At the present time the only report of human studies is that of Lewis and Taylor (4) who fed raw soybean meal to two nitrogen depleted adult men. Nitrogen retention was about 20% lower in the subjects fed raw soybean meal than in those fed autoclaved meal, although nitrogen digestibility was invariable. The authors concluded that these experiments indicated that soybeans are a poor source of protein unless adequately heated.

A prerequisite of a physiologically significant effect in the digestive tract brought about by proteinase inhibitors in soybeans is the formation of complexes between the proteolytic enzymes and the inhibitors, and the stabilization of these complexes suffi-

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ciently to give a certain inhibition of activity. Figarella et al. (5, 6) and Laskowski et al. (7) have demonstrated complex formation between Kunitz' soybean trypsin inhibitor (SBTI) and the two human trypsins (cationic and anionic). The work of Mallory and Trawis (8) demonstrated that proteolytic activities in homogenous preparations of human cationic and anionic trypsin, chymotrypsin I and II are moderately to severely inhibited by SBTI, and by the Bowman/Birk inhibitor from soybeans (BBI) as well as by the Lima bean inhibitor (LBI). In vivo, the effects of proteinase inhibitors in soybean products on protein digestion will be an overall effect of several inhibitors on the total enzyme mixture hydrolyzing protein in the digestive tract. Accordingly, the overall effect depends both on the pattern of inhibitors present in the beans and the pattern of enzymes in the digestive tract.

Our work addresses this complex situation. To enable quantitative studies to be carried out, human pancreatic juice was employed as a model for the digestive juice in the small intestine. Extracts of raw soybeans and purified inhibitors were added to the juice and the reduction of trypic, chymotryptic and total proteolytic activity measured. Identical experiments were carried out using pancreatic extracts from rats. The effects on the human enzymes were compared with the effects on the corresponding enzymes from rat, an experimental animal extensively used as a model system for man.

#### MATERIALS AND METHODS

*Human pancreatic juice (HPJ).* The human pancreatic juice (HPJ) was collected from the duodenum at the mouth of the pancreatic duct after intravenous administration of pancreozymin and secretin to give maximal enzyme secretion. The collection procedure was adapted from and supervised by Dr. Petersen.<sup>3</sup> The HPJ collected is contaminated with minor amounts of duodenal juice containing enterokinase which activates the pancreatic proenzymes (9, 10). Immediate cooling ( $0^{\circ}$ ), freezing in small aliquots within 1 to 2 hours (liquid  $N_2$ ), and storing at  $-70^{\circ}$  satisfactorily conserved the proteolytic activity. After

thawing, the juice was used at once without dilution or any other treatment. At  $0^{\circ}$  the proteolytic activity was stable for at least 12 hours. Total proteolytic activity in three collections, from three different normal subjects, varied due to variations in the water content. The activity per ml in the most dilute sample was 60% of that of the most concentrated sample. The relative activity of trypsin and chymotrypsin when measured as described below, was invariably found to be 0.4,

$$\left( \frac{\Delta T_{\text{trypsin}}}{\text{minute}} \right) / \left( \frac{\Delta T_{\text{chymotrypsin}}}{\text{minute}} \right).$$

In the present experiments all HPJ used was taken from the same batch.

*Rat pancreas extract (RPE).* The rats used were of the Wistar strain AF/Han/MØ/Han of both sexes, 150 to 200 g.<sup>4</sup> The animals were given free access to food and water until dissection of pancreas, at 0800, according to the method of Treadwell and Roe (11). Pancreata from 10 rats were freezeclamped, ground, and stored at  $-70^{\circ}$ . The enzyme preparation (RPE) was obtained as follows: 150 mg of the powdered frozen tissue was extracted in 9 ml of buffer (0.20 M Tris-HCl, 0.05 M CaCl<sub>2</sub>, pH 8.0 as measured at  $30^{\circ}$ ) in the presence of 1 ml of bovine trypsin solution (0.25 mg/ml in 10<sup>-3</sup> M HCl) at  $4^{\circ}$  for 24 hours in order to activate the proenzymes. This activation procedure provided maximal total proteolytic activity. (The amount of bovine trypsin used for activation is so small to be detected in any assay systems for the rat enzymes.) Proenzymes in the homogenate are not affected either by freezing or by storing at  $-70^{\circ}$ . At  $4^{\circ}$  activated proteolytic enzymes in RPE are stable for at least 6 hours. The total proteolytic activity in 10 different homogenates varied only slightly. As in human pancreatic juice, the relative activity of trypsin and chymotrypsin was 0.4.

*Soybean extracts (SBE).* A mixture of 25 different raw soybean varieties<sup>5</sup> were de-

<sup>3</sup> Dr. H. Petersen, Ullevål Hospital, Oslo, Norway.

<sup>4</sup> Scanbur A/S, Ly. Skensved, Denmark.

<sup>5</sup> Rat laboratory stock diet (SIEFF), Bjølsen Vale-

mslie, Oslo, Norway.

<sup>6</sup> From Ilonga Research and Training Institute, Tanzania. Detailed description of the varieties will be published elsewhere.

hulled and milled (80 mesh). The blended meal was used as a model of "typical" soybean meal. Solutions of proteinase inhibitors were obtained by gentle stirring of soybean meal in 0.9% NaCl (1:50 (w/v)) for 2 hours at 4° (1). The supernatants after centrifugation for 30 minutes at 10,000  $\times$  g (4°) were stored at -20°. No changes in inhibitor content were found after prolonged storage (approx. 1 year) at -20°, or after 38 hours at 4°. Before use in the assay systems all inhibitor solutions were diluted 1:2 with 0.9% NaCl.

**Purified Inhibitors.** Kunitz soybean trypsin inhibitor (SBTI)\* at a concentration of 0.125 mg/ml 10<sup>-3</sup> M HCl and Lima bean inhibitor (LBI),<sup>†</sup> at 0.063 mg/ml 10<sup>-3</sup> M HCl were used. LBI (M.W. ca. 9,000) was used because it is homologous to the compact, cystine-rich Bowman/Birk soybean inhibitor (BBI) which is not commercially available.

**Protease and esterase assays.** Total proteolytic activity was measured with casein as substrate, using a modification of the method described by Kunitz (12). The casein concentration was increased from 0.5 to 1.0% to obtain a close-to-linear relationship between enzyme activity and enzyme concentration (13). In this assay system (2.0 ml), 20  $\mu$ l HPJ gave the same proteolytic activity (OD<sub>280</sub> ~ 0.7) as 55  $\mu$ l RPE. Consequently, these volumes of HPJ and RPE were also employed in the following assays of individual enzymes.

The activities of trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and carboxypeptidase A (CPA) (EC 3.4.12.2), and carboxypeptidase B (CPB) (EC 3.4.12.3) in the enzyme mixtures were measured (14, 15) using N-benzoyl-L-arginine-ethyl-ester (BAEE),<sup>‡</sup> N-benzoyl-L-tyrosine-ethyl-ester (BTEE),<sup>§</sup> N-carbobenzoxy-glycyl-phenylalanine (CGP),<sup>||</sup> and N-hippuryl-arginine (HA),<sup>||</sup> respectively, as substrates. Linear relationships between the activity of each enzyme and its concentration were obtained.

Inhibition of each enzyme was measured when 10 to 200  $\mu$ l of SBE, 0 to 25  $\mu$ g SBTI, or 0 to 12.5  $\mu$ g LBI were added to the incubation system (3.0 ml). A specific inhibitor of carboxypeptidase A and B (16), 1,10-ortho-phenanthroline,<sup>||</sup> was used to

characterize the enzyme composition of HPJ. Zero to ten  $\mu$ g were added per 20  $\mu$ l HPJ, and the effects on CPA and CPB were assayed separately. In the Kunitz' procedure (13), excess 1,10-*o*-phenanthroline was removed by chloroform extraction (3  $\times$  5 ml) before the spectrophotometric determination of "total proteolytic activity." Heat inactivated HPJ or RPE were obtained by heating 1 ml of the enzyme solution for 10 minutes at 100°.

The results were analyzed by two-way analysis of variance. Comparison among class means was carried out according to Snedecor and Cochran, (treatments, SBTI, LBI, and SBE, blocks, each level of inhibitor added, EMS = error of mean square). The minimum level of statistical significance accepted was  $P < 0.05$  (17).

## RESULTS

The effects of proteinase inhibitors were studied in three series of incubation mixtures, in which total proteolytic activity, trypsin and chymotrypsin activity in human pancreatic juice (HPJ), and rat pancreatic extract (RPE) were determined separately (figs. 1A to 1C and 2A to 2C, respectively). Significant inhibition by the three inhibitor preparations was demonstrated in all assay systems (figs. 1A to 1C, 2A to 2C). The inhibition of trypsin in RPE (fig. 2B) by LBI and SBE did not differ significantly. In the remaining five assay systems SBTI, LBI, and SBE gave different inhibition. These experiments demonstrate that proteinase inhibitors from soybeans effectively inhibit the human proteolytic enzymes. In the presence of a soybean extract (SBE) corresponding to 1.3 mg of soybean meal there was 50% inhibition of the total proteolytic activity in 20  $\mu$ l of HPJ (fig. 1A), and almost complete inhibition of the trypsin and chymotrypsin activities (figs. 1B, 1C).

The total proteolytic activity of the rat

\* Sigma type I-S no T-9003, Sigma Chemical Co., St. Louis, Missouri.

† Sigma type II-L no T-9378, Sigma Chemical Co., St. Louis, Missouri.

‡ Serva no 14000, Serva GmbH, Heidelberg, Germany.

§ Serva no 14790, Serva GmbH, Heidelberg, Germany.

|| Merck art. 2345, E. Merck, Darmstadt, Germany.

|| Merck art. 24556, E. Merck, Darmstadt, Germany.

|| Merck art. 7225, E. Merck, Darmstadt, Germany.

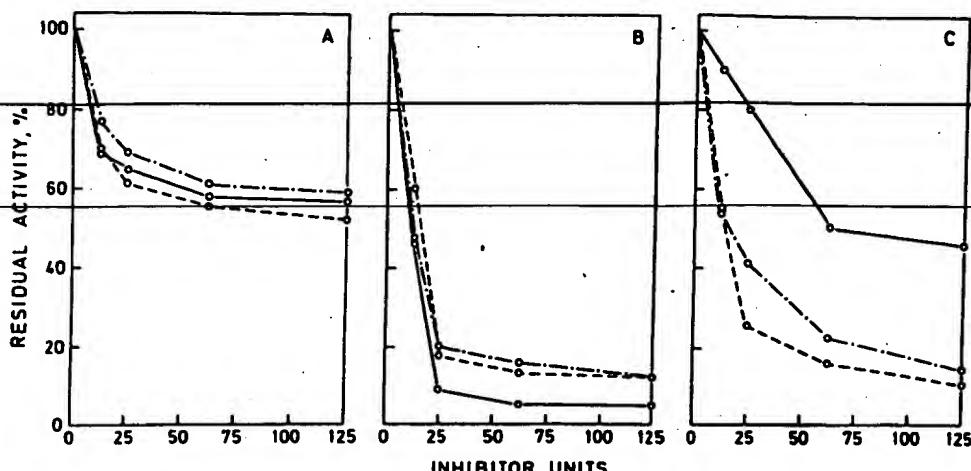


Fig. 1. Inhibition of total proteolytic activity (A), trypic activity (B), and chymotryptic activity (C) in human pancreatic juice (HPJ) by Kunitz' soybean trypsin inhibitor (SBTI) (—), lima bean inhibitor (LBI) (---), and soybean extracts (SBE) (-·-·). Units:  $10^{-3}$  nmole SBTI,  $10^{-3}$  nmole LBI,  $1.6 \mu\text{l}$  SBE (corresponding to  $6.8 \mu\text{g}$  soybean meal extracted). A:  $20 \mu\text{l}$  human pancreatic juice (HPJ) in  $2.0 \text{ ml}$  1% casein solution (12). TCA-soluble material was determined from change in absorbance at  $280 \text{ nm}$ . EMS = 3.1. B:  $20 \mu\text{l}$  HPJ in  $3.0 \text{ ml}$  BAEE solution (13). Benzoyl-arginine release was measured at  $253 \text{ nm}$ . EMS = 2.1. C:  $20 \mu\text{l}$  HPJ in  $3.0 \text{ ml}$  BTEE solution (13). Benzoyl-tyrosine release was measured at  $256 \text{ nm}$ . EMS = 1.7.

enzymes (fig. 2A) was inhibited by SBE to a somewhat lesser extent than that of the human enzymes (fig. 1A). The inhibition of rat trypsin and chymotrypsin by SBE was marked (figs. 2B, 2C) and similar in magnitude to that of the corresponding human enzymes (figs. 1B, 1C). This strong inhibition of trypsin and chymotrypsin from both sources is only partly reflected in the inhibition of total proteolytic activity.

The inhibition obtained with LBI resembled that of SBE in all assay systems. The differences between the pattern of inhibition with human and rat enzymes by SBE are to a large extent reproduced by LBI. However, the amount of soybean meal required to cause a given degree of inhibition is 100 times that of crystalline LBI.

Human proteinases seem to be inhibited less by SBTI than by the same amount (moles) of LBI (figs. 1A to 1C). With rat enzymes the picture is more complicated. In RPE, trypsin was inhibited more by SBTI than chymotrypsin was. SBTI and LBI had similar effects on the trypic activity of RPE (fig. 2B), whereas SBTI had a much weaker effect on chymotrypsin than

LBI had (fig. 2C). Differences in the pattern of inhibition of human and rat enzymes seem to be dependent on the type of inhibitor as well as the amount of inhibitor.

The relative inhibitory capacity of SBTI and LBI

$$\left( \frac{\Delta \text{activity}}{\text{mole SBTI}} \right) / \left( \frac{\Delta \text{activity}}{\text{mole LBI}} \right)$$

towards rat trypsin differed markedly from that towards chymotrypsin. The relative inhibitory capacity of SBTI and LBI towards human trypsin and chymotrypsin was almost the same. This indicates a difference in the properties of the corresponding enzymes from man and rat.

LBI was used as a representative of the cystine-rich low molecular weight proteinase inhibitors. The inhibitory pattern of SBE appeared to be different from SBTI and similar to that of LBI. This becomes particularly clear in figures 2B and 2C. This indicates that the predominant inhibitors in SBE may be homologous to LBI and belong to the heat stable type.

When  $1.6 \mu\text{l}$  of LBI was added to the standard incubation mixtures contain-

ing HPJ, virtually no trypsin or chymotrypsin activity could be demonstrated, although approximately 40% of the total proteolytic activity was present (point  $\Delta$  in fig. 3). The importance of carboxypeptidase activity in this situation was tested in experiments using the zinc-chelating agent 1,10-*o*-phenanthroline. Figure 4 shows that the removal of zinc ions by this agent affects the activity of the two carboxypeptidases differently. When 1,10-*o*-phenanthroline was added to HPJ in which all the trypsin and chymotrypsin activity had been inhibited by 1.6 nmole of LBI, a further 25% of the total proteolytic activity was lost (fig. 3). The lowered residual activity, due to increasing amounts of 1,10-*o*-phenanthroline, was significantly different at each level of inhibitor added.

Carboxypeptidases contribute a significant part of both the total proteolytic activity in HPJ and the activity after inhibition with LBI. The remaining 15% of proteolytic activity after inhibition with LBI and 1,10-*o*-phenanthroline is probably due to elastase which is not affected by the inhibitors used.

## DISCUSSION

Previous findings that human proteinases were apparently little affected by soybean inhibitors prompted speculation that these inhibitors had very little relevance to human nutrition (18-20). The present work indicates that SBTI has a considerable inhibitory capacity towards human trypsin and chymotrypsin in HPJ (figs. 1A to 1C). Evidently protein digestion may be impaired by SBTI. The inhibition obtained using SBE indicates that soybean proteinase inhibitors, in general, may be of significance in human nutrition. Extracts of 70 g soybean meal were able to eliminate trypsin and chymotrypsin activity in 1 liter of HPJ. Therefore, extracts from about 100 g of raw soybean meal will be required to inhibit the trypsin and chymotrypsin secreted in 24 hours. Inhibitor patterns in soybeans are not well known. However, 25 different soybean varieties were mixed to obtain SBE, and it seems unlikely that only a few varieties should be responsible for the inhibitory character shown in all assay systems to be similar to LBI. Consequently, the low-molecular-weight and heat-stable inhibitor types may frequently be present.

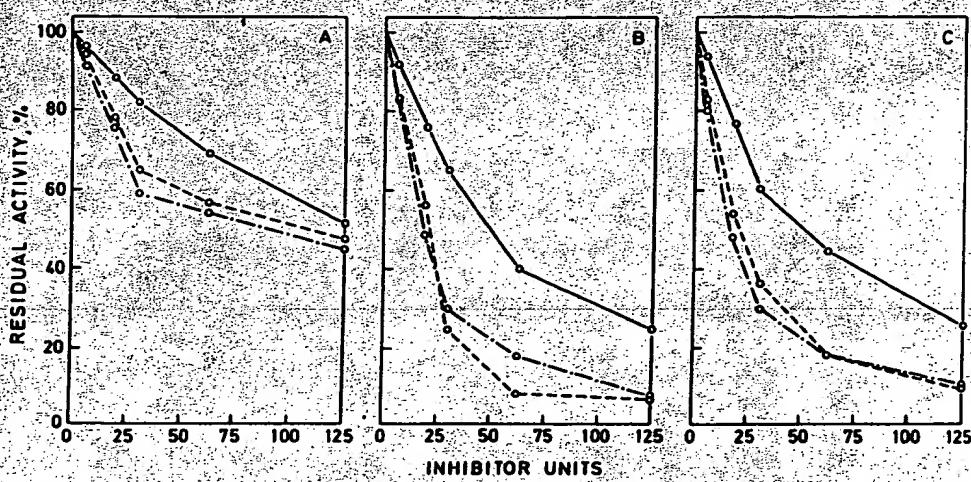


Fig. 2. Inhibition of total proteolytic activity (A), tryptic activity (B), and chymotryptic activity (C) in rat pancreas extract (RPE) by Kunitz soybean trypsin inhibitor (SBTI) (—), lima bean inhibitor (LBI) (---), and soybean extracts (SBE) (—·—). Units:  $10^{-4}$  nmole SBTI,  $10^{-4}$  nmole LBI,  $1.8 \mu\text{l}$  SBE (corresponding to  $6.8 \mu\text{g}$  soybean meal extracted). A:  $55 \mu\text{l}$  rat pancreatic extract (RPE) in  $2.0 \text{ ml}$  1% casein solution (12). TCA-soluble material was determined from change in absorbance at  $280 \text{ nm}$ . EMS = 1.2. B:  $55 \mu\text{l}$  RPE in  $3.0 \text{ ml}$  BAEE solution (13). Benzoyl-arginine release was measured at  $253 \text{ nm}$ . EMS = 1.6. C:  $55 \mu\text{l}$  RPE in  $3.0 \text{ ml}$  BTEE solution (13). Benzoyl-tyrosine release was measured at  $256 \text{ nm}$ . EMS = 2.4.

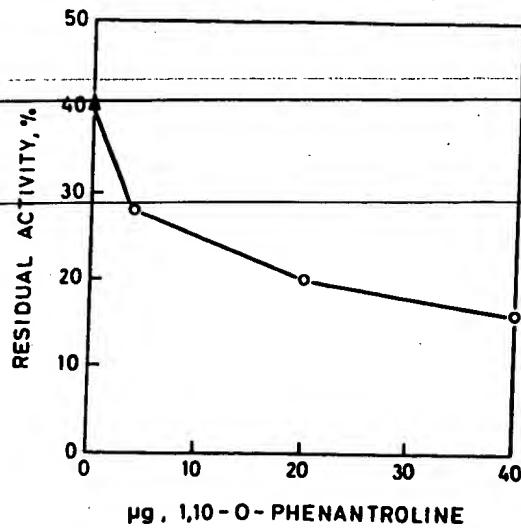


Fig. 3 Reduction in total proteolytic activity in human pancreatic juice (HPJ) due to inhibition of carboxypeptidase A and B by 1,10-o-phenanthroline. Two milliliters "standard" casein solution containing 20  $\mu$ l HPJ was made "trypsin and chymotrypsin free" by addition of 1.6 n mole LBI and residual proteolytic activity due to carboxypeptidase A and B further inhibited by 1,10-o-phenanthroline, 4 to 40  $\mu$ g (15). EMS = 1.7.

The overall effects as seen in the assay of total proteolytic activity will be influenced by the inhibitor pattern and the enzyme mixture present. The inhibitors will alter the relative active enzyme concentrations and this will, in turn, alter the relative effects of the inhibitors (8). In most feeding experiments with rats raw soybeans cause enlargement of the pancreas, reduced growth, reduced protein digestibility, impaired fat absorption, lowered energy utilization, and reduced availability of amino acids, vitamins, and minerals (2). The present work shows that an extract of approximately 1 g soybean meal eliminates the trypsin and chymotrypsin activity from one rat pancreas (approx. 0.55 g) while it reduces the total proteolytic activity to about 50% (figs. 2A to 2C). A substantial part of the proteolytic activity in HPJ is shown to be due to carboxypeptidase A and B (fig. 3). In the experiments where residual proteolytic activity in LBI-treated HPJ was studied, about 25% of the total activity could be accounted for by the carboxypeptidases. The remaining activity is assumed to be due to elastase. Soybeans

are not known to contain inhibitors of the carboxypeptidases, but elastase inhibitors have been identified (21). Consequently, the endopeptidase activity in general may be reduced while the exopeptidases may be unaffected by soybean extracts.

Secretion of proteolytic enzymes from the pancreas is under humoral regulation. Pancreozymin (cholecystokinin) is the main regulating hormone. Among other stimuli, the levels of active trypsin and chymotrypsin in the duodenum are important. Low levels of active enzymes stimulate pancreozymin secretion, elevate plasma pancreozymin and hence induce proteinase secretion. Proteinase inhibitors effectively lower the level of active intestinal trypsin and chymotrypsin. Consequently, the output of proteolytic enzymes from pancreas

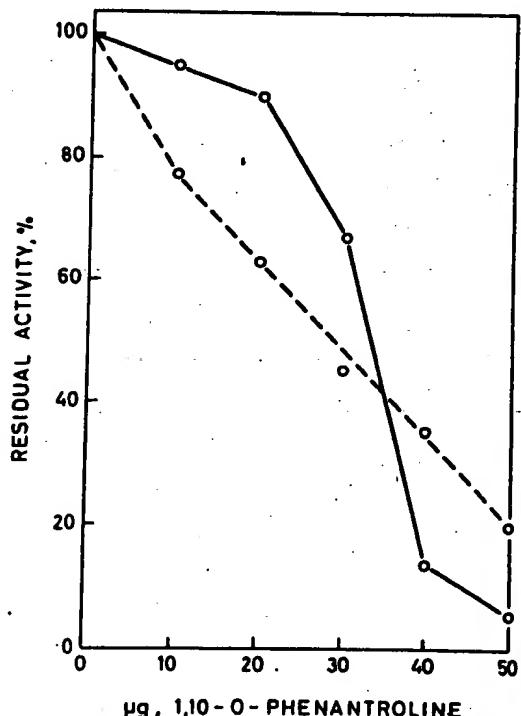


Fig. 4 Inhibition of carboxypeptidase A (CPA) and B (CPB) by 1,10-o-phenanthroline. A 3.0 ml incubation solution (15) containing 100  $\mu$ l HPJ was used. CPA activity was measured as amidase activity by determining the reduced absorbance at 232 nm using N-carbobenzoxy-glycyl-phenylalanine as substrate. CPB activity was determined at 254 nm using N-hippurylarginine as substrate. CPA, EMS = 2.0. CPB, EMS = 1.8 (CPA, O—O; CPB, O---O).

Truscheit, E., eds.), pp. 597-611, Springer-Verlag, Berlin-Heidelberg-New York.

8. Mallory, P. A. & Travis, J. (1975) Inhibition spectra of the human pancreatic endopeptidases. *Am. J. Clin. Nutr.* **28**, 823-830.
9. Petersen, H., Berstad, A. & Myren, J. (1973) Comparison of secretin and cholecystokinin in the diagnosis of pancreatic disease. *Rendic. Gastroenterol.* **5**, 174-181.
10. Petersen, H. (1974) Sekretintesten. *Tidsskr. Den Norske Lægeforening*, No 8, 509-513.
11. Treadwell, C. R. & Roe Jr., J. H. (1954) Technique for complete pancreatectomy in the rat. *Proc. Soc. Exp. Biol. Med.* **88**, 878-881.
12. Kunitz, M. (1947) Crystalline soybean trypsin inhibitor. II. General properties. *J. Gen. Physiol.* **30**, 291-310.
13. Kakade, M. L., Simons, N. R. & Liener, I. E. (1969) An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chem.* **46**, 518-526.
14. Rick, W. (1974) Chymotrypsin. In: *Methods of Enzymatic Analysis* (Bergmeyer, H. V., ed.), Vol. 2, pp. 1006-1012, Academic Press, New York.
15. Rick, W. (1974) Trypsin. In: *Methods of Enzymatic Analysis* (Bergmeyer, H. V., ed.), Vol. 2, pp. 1013-1017, Academic Press, New York.
16. Felber, J. P., Coombs, T. L. & Vallee, B. L. (1962) The mechanism of inhibition of carboxypeptidase A. *Biochemistry* **1**, 231-238.
17. Snedecor, G. W. & Cochran, W. G. (1967) *Statistical Methods*, p. 299, The Iowa State University Press, Ames, Iowa.
18. Feeney, R. E., Means, G. M. & Bigler, J. C. (1969) Inhibition of human trypsin, plasmin and thrombin by naturally occurring inhibitors of proteolytic enzymes. *J. Biol. Chem.* **244**, 1957-1960.
19. Travis, J. & Roberts, R. C. (1969) Human trypsin isolation and physical-chemical characterization. *Biochemistry* **8**, 2884-2889.
20. Coan, H. M. & Travis, J. (1971) Interaction of human pancreatic proteinases with naturally occurring proteinase inhibitors. In: *Proc. Int. Conf. Proteinase Inhibitors, Munic, Nov. 1970* (Fritz, H. & Tschesche, H., eds.), pp. 294-298, Walter de Gruyter, Berlin-New York.
21. Bieth, J. & Frechin, J.-C. (1974) Elastase inhibitors as impurities in commercial preparations of soybean trypsin inhibitor (Kunitz). In: *Proteinase Inhibitors, Proceedings of the 2nd International Research Conference* (Fritz, H., Tschesche, H., Greene, L. J. & Truscheit, E., eds.), pp. 291-304, Springer-Verlag, Berlin-Heidelberg-New York.
22. Chernic, S. S., Lepkowsky, S. & Chaikoff, I. L. (1948) A dietary factor regulating the enzyme content of the pancreas: Changes induced in the size and proteolytic activity of the chick pancreas by the ingestion of raw soybean meal. *Am. J. Phys.* **155**, 33-41.
23. Schneeman, B., Olds & Lyman, R. L. (1975) Factors involved in the intestinal feed-back regulation of pancreatic enzyme secretion in the rat. *Proc. Soc. Exp. Biol. Med.* **148**, 897-903.
24. Khayambashi, H. & Lyman, R. L. (1966) Stimulation of perfused pancreas secretion by plasma of rats fed soybean trypsin inhibitor. *Federation Proc.* **25**, 676.
25. Fölsch, V. R., Winkler, K. & Wormsley, K. G. (1974) Effect of a soybean diet on enzyme content and ultrastructure for the rat exocrine pancreas. *Digestion* **11**, 161-171.
26. Johnsen, L. R. (1976) The trophic action of gastrointestinal hormones. *Gastroenterology* **70**, 278-288.
27. Barnes, R. H. & Kwong, E. (1965) Effect of soybean trypsin inhibitor and penicillin on cystine biosynthesis in the pancreas and its transport as exocrine protein secretion in intestinal tract of the rat. *J. Nutr.* **86**, 245-252.
28. Frost, A. B. & Mann, G. V. (1966) Effects of cystine deficiency and trypsin inhibitor on the metabolism of methionine. *J. Nutr.* **89**, 49-54.
29. Nitsan, L. & Liener, I. E. (1976) Studies of the digestibility and retention of nitrogen and amino acids in rats fed raw or heated soy flour. *J. Nutr.* **106**, 292-305.
30. Matthews, D. M. (1975) Absorption of peptides by mammalian intestine. In: *Peptide Transport in Protein Nutrition* (Matthews, D. M. & Payne, J. W., eds.), pp. 61-146, North Holland Publishing Company, Amsterdam.
31. Adibi, S. A. & Mercer, D. W. (1973) Protein digestion in human intestine as reflected in luminal, mucosal and plasma amino acid concentrations after meals. *J. Clin. Invest.* **52**, 1586-1594.

## Effect of Dietar and Growth Re

**ABSTRACT** A experiments to det- ments of 1- to 21- fat milk basal diet ning at 1 day of a (PCV) of 21-day (ppm) of dietary. The heaviest 21-d Variations were fo on body weight. T by 21-day old chi requirement of 80 of 21-day old broil than a 10:1, 15:1, requirements for 1 same; however, th two criteria. J.

## INDEXING KEY hematological

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# Structure-Function Relationships of Proteinase Inhibitors from Soybean (Bowman-Birk) and Lima Bean

## MODIFICATION BY N-ACETYLIMIDAZOLE\*

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Contributions of tyrosyl residues to trypsin- and chymotrypsin-inhibitory activities in two homologous proteinase inhibitors were investigated by modifying them with *N*-acetylimidazole under various conditions. In Bowman-Birk soybean proteinase inhibitor, Tyr 55, immediately following the antichymotryptic site, Leu 53-Ser 54, is relatively inaccessible to *N*-acetylimidazole and can only be acetylated in the presence of 6 M guanidine hydrochloride but not in 8 M urea. The acetylation of Tyr 55 is accompanied by 60% loss in antichymotryptic activity. Deacetylation with hydroxylamine restores the activity to the original level. Tyr 69, located in the antitrypsin portion of the inhibitor, is exposed relatively to *N*-acetylimidazole and can be acetylated without denaturing agent. The acetylation of Tyr 69 parallels decrease in antitryptic activity. The inhibitor acetylated at Tyr 69 is fully active toward chymotrypsin and has 30 to 40% antitryptic activity of the native. The original level of antitryptic activity is restored upon deacetylation.

Tyr 69 of lima bean proteinase inhibitor is relatively inaccessible to *N*-acetylimidazole: 75% acetylation in the presence of 6 M guanidine hydrochloride and 17% without the denaturing agent. The acetylated inhibitor is fully active toward chymotrypsin but retains only 29% (acetylated without guanidine hydrochloride) and 17% (acetylated with guanidine hydrochloride) of the original antitryptic activity. Deacetylation partially restores the lost antitryptic activity in the inhibitor acetylated without the denaturing agent.

The total and irreversible loss of antitryptic activity in samples acetylated in the presence of 8 M urea or 6 M guanidine hydrochloride is attributed to the acetylation at the  $\epsilon$ -amino group of Lys 26 at the trypsin-inhibitory site.

Bowman-Birk soybean proteinase inhibitor and lima bean proteinase inhibitor are "double-headed" inhibitors which inhibit trypsin and chymotrypsin at independent reactive sites (1-6). A close homology between the inhibitors can be recognized by a near-identity in their amino acid sequences; only six positions are substituted from residues 13 to 73<sup>1</sup> (5, 7, 8).

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<sup>1</sup> In order to simplify presentation, amino acid sequences of the two inhibitors are aligned and residue numbers of LBI are used for both. Therefore, the amino acid sequence of BBI starts with residue 11 (8).

BBI<sup>2</sup> can be cleaved into three fragments by cyanogen bromide treatment followed by pepsin digestion. One fragment, residues 11 to 37 and 67 to 77 held together by four disulfide bonds, contains the trypsin-inhibiting site Lys 26-Ser 27. It has 84% antitryptic activity of the native and no antichymotryptic activity. The second consists of residues 38 to 66, which include the chymotrypsin-inhibitory site Leu 53-Ser 54 and three disulfide bonds. The antichymotryptic fragment possesses no antitryptic activity and 16% antichymotryptic activity of the intact inhibitor. The third fragment is a tetrapeptide from the COOH terminus of the native inhibitor (9). Spectrophotometric study of BBI showed that one of two tyrosyl phenolic groups is relatively exposed and can be *O*-acetylated by 100- to 1500-fold molar excess of *N*-acetylimidazole. The other is inaccessible to the reagent even in the presence of 8 M urea, but can be acetylated in the presence of 6 M guanidine hydrochloride. Which of the 2 tyrosyl residues is exposed could not be determined (10). LBI contains only one tyrosine, Tyr 69, which is relatively inaccessible to *N*-acetylimidazole. A 100-fold excess of the reagent in the presence of 6 M guanidine hydrochloride elicited about 75% *O*-acetylation (11).

Radiation studies of BBI and LBI suggest that damage to BBI Tyr 55, adjacent to the antichymotrypsin site, leads to loss of chymotrypsin-inhibitory activity. Radiation damage to Tyr 69, located in the antitryptic fragment, has no effect on either of the antiproteinase activities in both inhibitors (11, 12).

In the present study, antiproteinase activities of BBI and LBI derivatives acetylated under various conditions have been determined and the roles of tyrosyl residues in the activities have been evaluated.

## EXPERIMENTAL PROCEDURES

BBI and LBI were purified as described (10, 11). Acetylation and deacetylation of the inhibitors were performed according to reported methods (13, 14). Unreacted *N*-acetylimidazole was removed by dialysis against water at 4°C. Inhibitory activities of the inhibitors were measured spectroscopically, as described (12).

## RESULTS

Fig. 1 presents relationship between the *O*-acetylation of tyrosyl side chains and the change in inhibitory activities in BBI. The treatment of the inhibitor with increasing amounts of *N*-acetylimidazole resulted in a progressive acetylation of tyrosyl side chains. However, only 1 of 2 tyrosyl residues in BBI could be modified by 100- to 1500-fold molar excess of the reagent. Urea (8 M) did not affect the tyrosine acetylation, as one tyrosine remained unmodified by its presence (Table I). When BBI was acetylated in the presence of 6 M guanidine

<sup>2</sup> The abbreviations used are: BBI, Bowman-Birk soybean proteinase inhibitor; LBI, lima bean proteinase inhibitor.

hydrochloride, however, both tyrosines were almost completely acetylated (Table I).

Antichymotrypsin activity of BBI was not affected by the acetylation of the "accessible" tyrosine, as inhibitor modified by *N*-acetylimidazole alone was fully active toward chymotrypsin, even after treatment with 1500-fold molar excess of the reagent (Fig. 1). Antitrypsin activity, however, paralleled the acetylation of the accessible tyrosine and decreased as the tyrosyl side chain was acetylated. At 100-fold molar excess of the reagent, antitrypsin activity was about 40% of the original; further increase did not reduce the trypsin-inhibitory activity much. At 1500-fold excess, modified BBI retained about 25% antitryptic activity of the native. The lost antitryptic activity of the modified inhibitor acetylated without denaturing agent could be recovered fully by deacetylation of the *O*-acetylated tyrosyl side chain with hydroxylamine (Table I).

Although essentially no enhancement in tyrosine acetylation was observed in samples modified in the presence of 8 M urea, antitryptic activity was completely lost by this treatment, while antichymotryptic activity remained relatively unaffected (Table I). Deacetylation of the sample acetylated in 8 M urea restored the original antichymotryptic activity, but not the antitryptic activity.

A sample acetylated in the presence of 6 M guanidine hydrochloride and with both tyrosyl side chains *O*-acetylated lost all of its antitryptic activity and more than half of antichymotryptic activity (Table I). Antitryptic activity could not be regained by deacetylating *O*-acetyltyrosines but antichymotryptic activity was fully restored by this process.

The acetylation of LBI with a 100-fold molar excess of *N*-acetylimidazole induced 17% modification of tyrosine (Table I). The modified LBI lost more than two-thirds of the original antitryptic activity but retained full activity toward chymotrypsin. The deacetylation of *O*-acetyltyrosine by hydroxylamine realized only a partial recovery of the lost antitryptic activity.

The presence of 6 M guanidine hydrochloride during acetylation enhanced the extent of tyrosine modification to 75% from 17% (Table I). An increased loss in the antitryptic activity was also noted, but the antichymotryptic activity was unaffected by the presence of the denaturing agent. The lost antitryptic activity could not be regained by the restoration of tyrosyl side chains by hydroxylamine.

The treatment of the inhibitors with only 8 M urea, 6 M guanidine hydrochloride, or hydroxylamine without *N*-acetylimidazole did not have any effect on either of the antiproteinase activities.

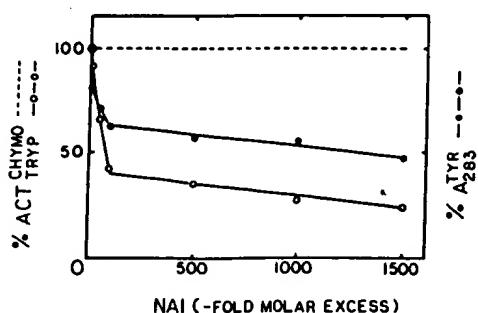


FIG. 1. Tyrosine acetylation and antiproteinase activities of BBI acetylated with *N*-acetylimidazole (NAI). ●—●, per cent of tyrosine acetylation using  $\Delta\epsilon_{225} = 2240$  for BBI with both tyrosyl side chains *O*-acetylated (10); ---, per cent change in antichymotryptic activity; ○—○, per cent change in antitryptic activity (12). Acetylation was performed in 50 mM sodium borate, pH 7.5, at 24°C for 2 h (10, 14).

TABLE I  
Per cent inhibitory activities of acetylated and deacetylated BBI and LBI

Acetylation was performed with 1500-fold (BBI) or 100-fold (LBI) molar excess of *N*-acetylimidazole with or without 8 M urea or 6 M guanidine hydrochloride. For deacetylation, protein solution buffered with 50 mM sodium phosphate, pH 7.5, was mixed with an equal volume of 2 M hydroxylamine preadjusted to pH 7.5 with 10 M sodium hydroxide (10, 14).

Molar excess <i>N</i> -acetylimidazole	Acetylated		Deacetylated		
	Per cent tyrosine acetylated	Antitrypsin	Antichymotrypsin	Antitrypsin	
Control BBI	0	100	100	100	100
1500	52	41	100	100	100
1500 + 8 M urea	50	0.4	85	1.7	100
1500 + 6 M guanidine hydrochloride	97	0.4	40	1.7	100
LBI					
100	17	29	100	36	96
100 + 6 M guanidine hydrochloride	75	17	103	17	103

#### DISCUSSION

Of 2 tyrosyl residues in BBI, Tyr 55 is located next to antichymotryptic site Leu 53-Ser 54. The other, Tyr 69, resides in the antitryptic fragment but the proximity of this tyrosine to the trypsin-inhibitory site is unknown (9). In LBI, Tyr 69 is the sole tyrosine, as position 55 has isoleucine in the inhibitor (15).

From results of the present study, the tyrosyl residue exposed to *N*-acetylimidazole in BBI may be assigned to Tyr 69 in the antitryptic fragment because: (a) the loss of antitryptic activity follows closely the acetylation of the accessible tyrosine, (b) antitryptic activity can be restored to the original level by deacetylating the BBI derivative which was acetylated without denaturing agent; and (c) the acetylation of the accessible tyrosine does not have any effect on antichymotryptic activity. The other tyrosyl residue that is relatively inaccessible to *N*-acetylimidazole must then be Tyr 55, adjacent to the antichymotryptic active site. Additional evidence is that the acetylation of the "inaccessible" tyrosine results in 60% reduction of antichymotryptic activity which can be restored upon deacetylation.

The acetylation of Tyr 69 in BBI without denaturing agent induces 60 to 70% loss in antitryptic activity which is fully recoverable by deacetylation. The presence of 8 M urea during the acetylation does not influence the degree of tyrosine modification, but it produces a complete and irreversible loss in antitryptic activity. The presence of 8 M urea must have promoted the acetylation of other amino acid residue(s) essential to trypsin inhibitory activity. Although *N*-acetylimidazole is used primarily for acetylation of tyrosyl side chains, acetylation of amino groups has been demonstrated as well (13).  $\epsilon$ -Amino groups of lysines can be modified by such a treatment (16), but only *O*-acetyltyrosine is deacetylated by the procedure used (13). The substitution of Lys 26 at the trypsin inhibitory site may then be suggested for the irreversible loss in antitryptic activity. Similarly, a total and irreversible loss of antitryptic activity in samples acetylated in the presence of 6 M guanidine hydrochloride may also be attributed to the irreversible acetylation of the same lysyl residue.

The results of LBI acetylation support the assignment of tyrosine residues in BBI: The acetylation of Tyr 69 in LBI, with or without 6 M guanidine hydrochloride, has no effect on antichymotryptic activity at all while antitryptic activity is

reduced. A part of the lost antitryptic activity in the sample modified without the denaturing agent can be recovered upon deacetylation. The irreversible acetylation of Lys 26 might also be assumed for unrecoverable loss of antitryptic activity in LBI.

A conclusion that modification of Tyr 55 results in the loss of antichymotryptic activity is in accord with a previous study using free radicals as modifying agents. In that study radiation damage to Tyr 55 was suggested to be accompanied by decline in antichymotryptic activity without affecting antitryptic activity (12). However, the modification of Tyr 69 by free radicals had no effect on either of the antiproteinase activities in both BBI and LBI (11, 12), a conclusion which seems contradictory to results of the acetylation study.

Since radiation and acetylation produce different products, the modification of the same residue in a protein by these two techniques may be expected to induce different effects on the functions. Derivatives of  $\text{Br}_2^-$  and  $(\text{CNS})_2^-$  attack on tyrosine have not been well characterized. By analogy with hydroxyl radicals, however, a major product of tyrosine degradation by these radical anions may be assumed to be 3,4-dihydroxy-phenylalanine (17-19), a minor structural modification. On the other hand, acetylation introduces a relatively bulky acetyl group at the hydroxyl group. Such a bulky substitution may sterically hinder the formation of a proper enzyme-inhibitor complex.

Although the modification of Tyr 55 in BBI by either radiation or acetylation results in the loss of antichymotryptic activity, it is not an "essential" amino acid and probably does not participate directly in chymotrypsin binding. Thus it is replaced by other amino acids in inhibitors homologous to BBI without losing antichymotryptic activity, isoleucine in LBI (15) and methionine in garden bean proteinase inhibitor

II (20). However, the integrity of Tyr 55 is essential to chymotrypsin-inhibitory activity in BBI.

#### REFERENCES

1. Birk, Y., Gertler, A., and Khalef, S. (1967) *Biochim. Biophys. Acta* 147, 402-404
2. Frattali, V., and Steiner, R. F. (1969) *Biochem. Biophys. Res. Commun.* 34, 480-487
3. Seidl, D. S., and Liener, I. E. (1971) *Biochim. Biophys. Acta* 251, 83-93
4. Seidl, D. S., and Liener, I. E. (1972) *Biochim. Biophys. Acta* 258, 303-309
5. Odani, S., and Ikenaka, T. (1972) *J. Biochem. (Tokyo)* 71, 839-848
6. Krahn, J., and Stevens, F. C. (1970) *Biochemistry* 9, 2646-2652
7. Odani, S., and Ikenaka, T. (1973) *J. Biochem. (Tokyo)* 74, 697-715
8. Stevens, F. C., Wuerz, S., and Krahn, J. (1974) in *Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L. J., and Truscheit, E., eds) pp. 344-354, Springer-Verlag, Berlin
9. Odani, S., and Ikenaka, T. (1973) *J. Biochem. (Tokyo)* 74, 857-860
10. Kay, E. (1976) *J. Biol. Chem.* 251, 3411-3416
11. Kay, E., and Gumbiner, B. (1979) *J. Biol. Chem.* 284, 7643-7647
12. Wandell, J. L., and Kay, E. (1977) *Radiat. Res.* 72, 414-426
13. Riordan, J. F., and Vallee, B. L. (1972) *Methods Enzymol.* 25B, 500-506
14. Kay, E., Strickland, E. H., and Billups, C. (1974) *J. Biol. Chem.* 249, 797-802
15. Tan, C. G. L., and Stevens, F. C. (1971) *Eur. J. Biochem.* 18, 515-523
16. Heller, J., and Horwitz, J. (1975) *J. Biol. Chem.* 250, 3019-3023
17. Rowbottom, J. (1955) *J. Biol. Chem.* 212, 877-885
18. Fletcher, G. L., and Okada, S. (1961) *Radiat. Res.* 15, 349-354
19. Wheeler, O. H., and Montalvo, R. (1969) *Radiat. Res.* 40, 1-10
20. Wilson, K. A., and Laskowski, M., Sr. (1974) in *Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L. J., and Truscheit, E., eds) pp. 286-290, Springer-Verlag, Berlin

## **CONTINUING EDUCATION/REVIEWS**

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## THE EFFECTS OF SOYBEAN TRYPSIN INHIBITORS ON THE PANCREAS OF ANIMALS AND MAN: A REVIEW

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The introduction of a new or alternative food source into the American diet often creates a myriad of questions regarding its safety and nutritional value. This is especially true when that food is composed of a complexity of components. Soybeans belong to this category. Most of the enzymes found in raw soybeans remain chemically active unless they are heated. A deleterious effect was noted on the pancreas in some species of animals fed raw soybeans. This was thought to be a result of those enzymes. For this reason, soybeans and their enzymatic components were scrutinized for any purported toxicity to man.

Inhibitors of proteolytic activity of many enzymes are found throughout the plant kingdom, particularly among the legumes. Table 1 lists the variety of plants containing protease inhibitors.

In addition to the legumes, cereal grains, grasses, potatoes and eggplant all contain protease inhibitors (1-3).

Humans have 2 types of trypsin, cationic and anionic, that are similar to mammalian trypsins in amino acid composition (4,5); however, only four disulfide bonds are possible, compared with six for other mammalian species (5).

The soybean inhibitors of trypsin (STI) in particular have received considerable attention because of their effects on animals in both growth and pancreatic activity (6-11). These effects include pancreatic hypertrophy in rats (1,7-11) and chicks (2,8,12), but not in dogs (12-15) or calves(14). Further studies in rats revealed not only hypertrophy and hyperplasia of the pancreas in rats fed continuous diets containing raw soya flour, but they also developed hyperplastic nodules and adenomas. A few of the rats developed pancreatic cancer (9). Growth inhibition and diarrhea were also seen in calves (6), rats (2), chicks (12) and mice (16).

At least five trypsin inhibitors have been isolated from soybeans (2) and soybean whey (17). According to Ikenaka et al (18), Kunitz isolated the first inhibitor in 1945; it is composed of a single polypeptide chain with approximately 200 amino acids and two disulfide bonds as shown in Figure 1.

Another trypsin inhibitor, the Bowman-Birk inhibitor, differs from the Kunitz inhibitor not only in the fact that it is a more potent

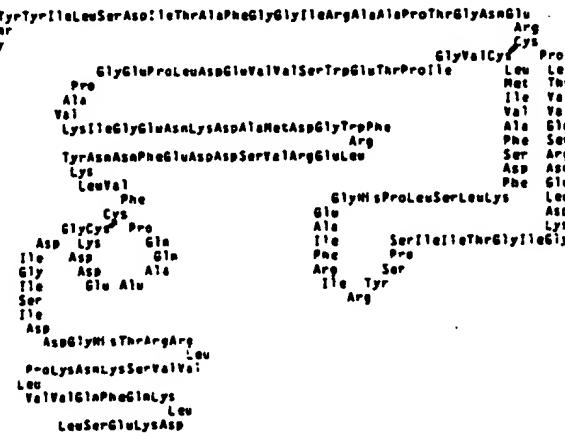


Fig 1. Kunitz Inhibitor

inhibitor of trypsin, but it also displays powerful inhibitory activity toward chymotrypsin. This inhibitor contains 17 disulfide bonds which account for its high resistance to severe treatment with heat, acid, alkali, pepsin or papain. The lima bean (Phaseolus lunatus) and other legumes also contain similar resistant trypsin inhibitors (2).

## MECHANISM OF ACTION OF INHIBITORS

Substantial evidence indicates that the feeding of raw soybeans and purified soy trypsin inhibitors accelerates protein synthesis in the pancreas and stimulates hypersecretion of pancreatic enzymes (amylase, lipase and trypsin) into the intestinal tract<sup>(1,7,8)</sup>. The secretory response of the pancreas to dietary trypsin inhibitors is an indirect response that is initiated in the intestine and not in the blood. In rats, pancreatic enzyme secretion is suppressed by negative feedback inhibition resulting from the presence of trypsin and chymotrypsin in the intestinal tract. The trypsin inhibitors increase pancreatic enzyme secretion by forming inactive trypsin-trypsin inhibitor complexes and therefore decrease the suppression exerted by free trypsin (1,11). Feedback inhibition occurs in humans and pigs, but not in dogs (1). The presence of protein and trypsin inhibitors in the duodenum results in a release of cholecystokinin (CCK) from the binding sites in the mucosa. CCK also has trypsin inhibiting activity and can cause pancreatic hypertrophy and inhibition of rat growth (1), as shown in Figure 2.

Table 1. Distribution of Disease Inhibitors in Plants

Botanical Name	Common Name	Part of Plant
<i>Arachis hypogaea</i>	Peanut, ground nut	Seed skin
<i>Artocarpus integrifolia</i>	Jack fruit	Seed
<i>Avena sativa</i>	Oats	Seed
<i>Beta vulgaris</i>	Beet, beetroot	Root
<i>Brassica rapa</i>	Turnip	Root
<i>Cajanus cajan</i>	Red gram	Seed
<i>Cajanus indicus</i>	Pigeon peas	Seed
<i>Canavalia ensiformis</i>	Jack bean	Seed
<i>Ceratonia siliqua</i>	Carob bean	Seed
<i>Cercis canadensis</i>	Redbud tree	Seed
<i>Chamaecrista fasciculata</i>	Partridge pea	Seed
<i>Cicer arietinum</i>	Bengal gram, chick pea, garbanzo	Seed
<i>Colocasia esculenta</i>	Taro	Root
<i>Cyanopsis psoraloides</i>	Guar bean	Seed
<i>Dolichos lablab</i>	Field bean, hyacinth bean	All parts
<i>Faba vulgaris</i>	Double bean	All parts
<i>Fagopyrum esculentum</i>	Buckwheat	Seed
<i>Gleditsia tricanthos</i>	Honey locust	Seed
<i>Glycine max</i>	Soybean	Seed
<i>Glymnocladus dioica</i>	Kentucky coffee bean	Seed
<i>Hordeum vulgare</i>	Barley	Seed
<i>Ipomoea batata</i>	Sweet potato, yam	Root and leaves
<i>Lactuca sativa</i>	Lettuce	Seed
<i>Lens esculenta</i>	Lentil	Seed
<i>Lespedeza stipulacea</i>	Lespedeza	Seed
<i>Mendicago sativa</i>	Alfalfa, lucerne	Leaf
<i>Mucuna deeringianum</i>	Florida velvet bean	Seed
<i>Oryza sativa</i>	Rice	Seed
<i>Phaseolus aureus</i>	Green gram, mung bean	Leaves
<i>Phaseolus coccineus</i>	Scarlet runner bean	Seed
<i>Phaseolus vulgaris</i>	Garden bean	Seed
<i>Phaseolus lunatus</i>	Lima bean	Seed

A casein diet is also a stimulant of pancreatic secretion in the rats by binding to trypsin during digestion with the result that feedback inhibition is decreased (1).

#### INHIBITOR SPECIES SPECIFICITY

Endogenous inhibitors to trypsin have been found to bovine trypsin, porcine trypsin and human trypsin. Human pancreatic secretory trypsin inhibitor effectively inhibits human

cationic and anionic trypsin, bovine trypsin and porcine trypsin. The cationic human trypsin is not inhibited by bovine or porcine trypsin inhibitors (4,19).

Trypsin inhibitors can discriminate between bovine and human trypsin (20). Soybean trypsin inhibitor can inhibit human trypsin 2 (anionic), 100% in a 1:1 molar ratio as it inhibits bovine trypsin; however, human trypsin 1 (cationic) is inhibited only 40% at a 1:1 molar ratio (4). The reaction of human trypsin with soybean trypsin inhibitor is shown in Figure 3.

Inhibitors known to possess a susceptible bond of type Arg-X (4) are among the poorest inhibitors of human trypsin, especially trypsin 1. The soybean trypsin inhibitor is classified in this group. The primary structure of the reactive site of modified STI is

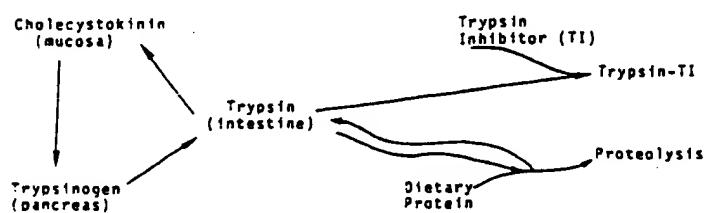


Fig 2. Regulation of trypsin secretion

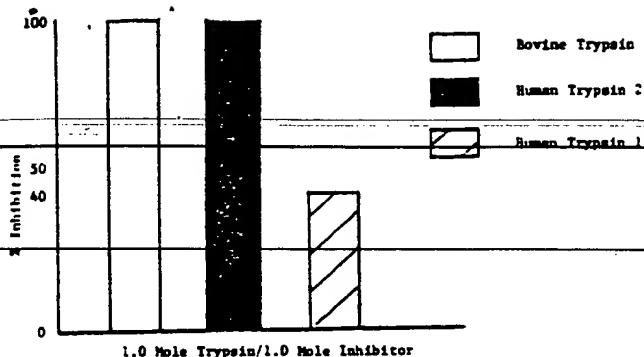


Fig 3. Inhibition of bovine and human trypsin with soybean trypsin inhibitor

shown below, the residues labeled according to the notation of SCHECHTER and BERGER (21):

P 4 P 3 P 2 P 1 : P 1 P 2 P 3 P 4  
Pro Ser Tyr Arg OH : H Ile Arg Phe He

The weak inhibition of human trypsin 1 by STI has never been described for the trypsin of any other mammalian species. In addition, the trypsin that fails to be inhibited by STI represents the major part of the potential trypsin activity of the whole juice (about 2/3) (4). Most authors found weak and poorly reproducible inhibition of human trypsin by STI (1,4,5,20,22). This is due to lesser stabilization of the trypsin-inhibitor complex (23).

#### TRYPSIN INHIBITORS AND CANCER

Unlike the effects of STI on man, rats developed pancreatic hypertrophy (1,2,7,9-11) which may act as a promoter for potential pancreatic carcinogens. Raw soya flour alone fed to rats led to the development of hyperplastic nodules, adenomas and, in some cases, cancer (9). Combining two known carcinogens, azaserine and di(2-hydroxypropyl)nitrosamine, with raw soya flour diets augmented their carcinogenicity in the pancreas of rats (24,25).

Mice fed diets containing raw soybeans, however, showed a delay in the appearance of tumors initiated with nitroquinoline-oxide (N O) by 45 days and a decrease in the number of tumors by 50% after 200 days (26).

#### SUMMARY

Human trypsin is more resistant to inhibition than is the trypsin of other mammalian species. The effect on human trypsin of soybean trypsin inhibitor in soy protein does not appear to be a potential hazard to man. Therefore, the elimination of STI does not seem to be necessary for humans. In animal diets, however, pancreatic toxicity must be considered whenever soybean protein is utilized. Soybeans should be treated to increase their nutritional benefits and decrease any animal health risks (27-29). This will insure healthy control subjects in laboratory situations and avoid misinterpretation of pathologic data. The treatment suggested is heat (2,18,25,30-32) since heat will destroy most of the soybean trypsin inhibitors. Ad-

ditional supplementation is required following heat treatment for amino acids (33,34) such as methionine, valine, and threonine; for choline (2,14,35); and for the minerals zinc (36) and calcium (11,34). Excessive heat must be avoided since it will decrease the nutritional value of soybean protein and increase lysinoalanine, a nephrotoxic substance (12). Finally, the use of STI as a promotor in the study of potential pancreatic carcinogens may prove beneficial for cancer research (24,25) and might be considered in the future.

#### REFERENCES

- Anderson, RL, Rackis, JJ, Tallent, WH: Biologically active substances in soy products. In *Soy Protein and Human Nutrition*, Academic Press, Inc, New York, pp. 209-233, 1979.
- Liener, I, Kakade, ML: *Toxic Constituents of Plant Foodstuffs*, Chapter 2, Academic Press, New York, 1969.
- Wilson, KA, Laskowski, M: The partial linear sequence of garden bean inhibitor I and location of the protease reactive sites. *Bayer Symposium V "Proteinase Inhibitors"*, pp. 286-290, 1974.
- Figarella, C, Negri, GA, Guy, O: Studies on inhibition of the two human trypsins. *Bayer Symposium V, "Proteinase Inhibitors"*, pp. 213-222, 1974.
- Travis, J, Roberts, RC: Human trypsin, isolation and physical-chemical characterization. *Biochemistry* 8:2884, 1969.
- Gorrill, ADL, Thomas, JW: Body weight changes, pancreas size and enzyme activity, and proteolytic enzyme activity and protein digestion in intestinal contents from calves fed soybean and milk protein diets. *J Nutr* 92: 215-223, 1967.
- Khayambashi, H, Lyman, RL: Secretion of rat pancreas perfused with plasma from rats fed soybean trypsin inhibitor. *Am J Physiol* 217: 646-651, 1969.
- Lyman, RL, Lepkovsky, S: The effect of raw soybean meal and trypsin inhibitor diets on pancreatic enzyme secretion in the rat. *J Nutr* 62: 269-284, 1957.
- McGinness, EE, Morgan, RGH, Levison, DA, Frape, DL, Hopwood, D, Wormsley, KG: The effects of long-term feeding of soya flour on the rat pancreas. *Scand J Gastroenterol* 15: 497, 1980.
- Rackis, JJ: Physiological properties of soybean trypsin inhibitors and their relationship to pancreatic hypertrophy and growth inhibition of rats. *Food Technol* 20: 1482, 1966.
- Rackis, JJ, McGhee, JE: Effects of soy proteins containing trypsin inhibitors in long term feeding studies in rats. *J Am Oil Chem Soc* 56: 162, 1979.
- Wolf, WJ, Cowan, JC: Soybeans as a Food Source. CRC Press, Inc, 18901 Cranwood Parkway, Cleveland, OH 44128, pp 51-53, 1975.
- Hooks, RD, Hays, WV, Speer, VC, McCall, JT: Effects of raw soybeans on pancreatic enzyme concentrations and performance of pigs. *Fed Proc Fed Am Soc Exp Biol* 24: 894, 1965.
- Patten, JR, Richards, EA, Pope, H: The effect

of raw soybean on the pancreas of adult dogs. *Proc Soc Exp Biol Med* 137: 58, 1971.

15. Patten, JR, Richards, EA, Wheeler, J: The effect of dietary soybean trypsin-inhibitor on the histology of dog pancreas. *Life Sci* 10, Part II: 145-250, 1971.

16. Schingoethe, DJ, Aust, SD, Thomas, JW: Separation of a mouse growth inhibitor in soybeans from trypsin inhibitors. *J Nutr* 100: 739, 1970.

17. Elridge, AC, Anderson, RI, Wolf, WJ: Polyacrylamide-gel electrophoresis of soybean whey proteins and trypsin inhibitors. *Arch Biochem Biophys* 115: 495-504, 1966.

18. Ikenaka, T, Odani, S, Koide, T: Chemical structure and inhibitory activities of soybean proteinase. *Bayer Symposium V, "Proteinase Inhibitors"*, pp. 325-343, 1974.

19. Greene, LJ, Roark, DE, Bartelt, DC: Human pancreatic secretory trypsin inhibitor. *Bayer Symposium V, "Proteinase Inhibitors"*, pp. 188-198, 1974.

20. Laskowski, M, Jr, Kato, I, Leary, TR, Schrode, J, Sealock, TW: Evolution of specificity of protein proteinase inhibitors. *Bayer Symposium V, "Proteinase Inhibitors"*, pp. 597-611, 1974.

21. Kowalski, D, Leary, TR, McKee, RE, Sealock, RW, Wang, D, Laskowski, M, Jr: Replacement, insertions, and modifications of amino acid residues in the reactive site of soybean trypsin inhibitor (Kunitz). *Bayer Symposium V, "Proteinase Inhibitors"*, pp. 311-324, 1974.

22. Bieth, J, Aubry, M: The interaction of human cationic trypsin and chymotrypsin II with human serum inhibitors. *Bayer Symposium V, "Proteinase Inhibitors"*, pp. 53-62, 1974.

23. Finkenstadt, WR, Hamid, MA, Mattis, JA, Schrode, J, Sealock, RW, Wang, D, Laskowski, M, Jr: Kinetics and thermodynamics of the interaction of proteinases with protein inhibitors. *Bayer Symposium V, "Proteinase Inhibitors"*, pp. 389-411, 1974.

24. Levison, DA, Morgan, RGH, Brimacombe, JS, Hopwood, D, Coghill, G, Wormsley, KG: Carcinogenic effects of di(2-hydroxypropyl)nitrosamine (DHPN) in male Wistar rats: Promotion of pancreatic cancer by a raw soya flour diet. *Scand J Gastroenterol* 14: 217, 1969.

25. Morgan, RGH, Levinson, DA, Hopwood, D, Saunders, JHB, Wormsley, KG: Potentiation of the action of azaserine on the rat pancreas by raw soya bean flour. *Cancer Lett* 3: 87-90, 1977.

26. Rossman, TG, Troll, W: Protease inhibitors in carcinogenesis: Possible sites of action. In *Carcinogenesis*, V. 5: *Modifiers of Chemical Carcinogenesis*. Ed, TJ Slaga, Raven Press, New York, 1980.

27. Kakade, ML, Rackis, JJ, McGhee, JE, Puski, G: Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. *Cereal Chem* 51: 376-382, 1974.

28. Longenecker, JB, Martin, WH, Sarett, HP: Improvement in the protein efficiency of soybean concentrates and isolates by heat treatment. *J Agric Food Chem* 12: 411-412, 1964.

29. Mustakas, GC, Albrecht, WJ, Bookwalter, GN, McGhee, JE, Kwolek, WF, Griffin, RL: Extruder processing to improve nutritional quality, flavor, and keeping quality of high-fat soy flour. *Food Technol* 24:1290, 1970.

30. Albrecht, WJ, Mustakas, GC, McGhee, JE: Rate studies on atmospheric steaming and immersion cooking of soybeans. *Cereal Chem* 43: 400-407, 1966.

31. Birk, Y, Gertler, A: Effect of mild chemical and enzymatic treatments of soybean meal and soybean trypsin inhibitors on their nutritive and biochemical properties. *J Nutr* 75: 379-387, 1961.

32. Borchers, R: Raw soybean growth inhibitor. *Fed Proc Fed Am Soc Exp Biol* 24: 1494-1497, 1965.

33. Borchers, R: Counteraction of the growth depression of raw soybean oil meal by amino acid supplements in weanling rats. *J Nutr* 75: 330-334, 1961.

34. Groot, AP, Slumpt, P: Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. *J Nutr* 98: 45-56, 1969.

35. Theuer, RC, Sarett, HP: Nutritional adequacy of soy isolated infant formulas in rats: Choline. *J Agric Food Chem* 18: 913, 1970.

36. O'Dell, BL, Savage, JE: Effect of phytic acid on zinc availability. *Proc Soc Exp Biol Med* 110: 417, 1962.

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THE SOLDIER had just arrived at a camp near his home after three years overseas and was very eager to be reunited with his wife. But try as he would, he could only get a two-hour leave. After a six-hour absence, he came back to camp. "Why are you four hours AWOL?" barked the sergeant. "Well," replied the soldier, "when I got home I found my wife in the tub, and it took me four hours to dry out my uniform."